

Award Number: W81XWH-12-1-0460

TITLE: *Öæ| ^ Ö^c & q } Á Á çæ ã Æ Öæ & ^ Á Á { [! Ö] ã @ ã { Ë / æ * ^ c å Á [| ^ & | æ Á d æ [~ } å*

PRINCIPAL INVESTIGATOR: Animesh Barua, Ph.D.

CONTRACTING ORGANIZATION: Rush University Medical Center
Chicago, IL 60612

REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 2013		2. REPORT TYPE Annual		3. DATES COVERED 30 Sep 2012 - 29 Sep 2013	
4. TITLE AND SUBTITLE Öæ Ö^c & q } Ä - Á ç ä ä Öæ & ! Ä ^ Á ~ { [! Ö] ä @ ä { Ë / æ * ^ c ä Á [^ & æ Á d æ [~ } ä				5a. CONTRACT NUMBER W81XWH-12-1-0460	
				5c. PROGRAM ELEMENT NUMBER	
				5d. PROJECT NUMBER	
6. AUTHOR(S) Animesh Barua, Ph.D. E-Mail: Animesh_Barua@rush.edu				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
				5g. PROJECT ELEMENT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Rush University Medical Center, Chicago, IL 60612				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The high rate of death of ovarian cancer (OVCA) patients can be prevented if it is detected at early stage. Unfortunately, currently available traditional transvaginal ultrasound (TVUS) imaging together with serum CA-125 levels cannot detect OVCA at early stage. Malignant nuclear transformations followed by the establishment of tumor associated neo-angiogenesis are the early events in tumor development. Ovulation is an inflammatory process which exposes ovarian surface and fimbrial epithelium to inflammatory factors including interleukin 16 (IL-16). Inflammation of the ovary and tubal epithelium due to frequent ovulation leads to the development of oxidative stress and longstanding unresolved oxidative stress causes malignant transformation. Expression of IL-16 by the tumor epithelium and its serum levels has been reported to be increased during OVCA development. Thus IL-16 represents a potential marker of early OVCA which can be detected <i>in vivo</i> by ultrasound imaging provided an IL-16-targeted molecular imaging agent can be developed. The goal of this study is to develop and test the efficacy of molecular (IL-16)-targeted ultrasound (MT-U/S) imaging probe for the detection of early OVCA. This goal is being accomplished by two specific aims. The results of Aim-1 suggest that IL-16-targeted imaging probes improved the visualization and detection of ovarian tumors in laying hens. These results will be used in Aim-2 to test the efficacy of IL-16-targeted MT-U/S imaging probes in detecting early OVCA in laying hens in a prospective study.					
15. SUBJECT TERMS Ovarian cancer, Early detection, Molecular-targeted Ultrasound imaging, IL-16, laying hen model					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 16	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	12
Reportable Outcomes.....	12
Conclusion.....	13
References.....	13
Appendice.....	15

INTRODUCTION:

Ovarian cancer (OVCA) is a fatal malignancy of women with high case-to-death ratio of patients [1]. This high rate of death can be prevented if it is detected at early stage. Unfortunately, non-specificity of symptoms at early stage and a lack of an effective early detection test, OVCA in most cases is detected at late stages when the 5-year survival rate of patients is <20% as opposed to >80% if detected at early stage [2]. Ovulation is an inflammatory process which exposes ovarian surface (site of ovulatory rupture) and fimbrial epithelium to inflammatory factors including interleukin 16 (IL-16) secreted by immune cells. Inflammation of the ovary and tubal epithelium due to frequent ovulation leads to the development of oxidative stress and longstanding unresolved oxidative stress has been suggested to cause malignant transformation. Malignant nuclear transformations followed by the establishment of tumor associated neo-angiogenesis are the early events in tumor development and progression. During malignant nuclear transformation, the shape and sizes of the nucleus undergo profound changes together with the rearrangement of nuclear matrix proteins (NMP) leading to the shedding of NMPs into the circulation. Anti-NMP antibodies are produced in response to shed NMPs [3, 4]. On the other hand, expression of IL-16 by the tumor epithelium and its serum levels increase in association with ovarian tumor development [5]. Thus IL-16 represents a potential marker of early OVCA which can be detected by ultrasound imaging provided an IL-16-targeted MT-U/S imaging agent can be developed. Approaches involving serum CA-125 levels, traditional transvaginal ultrasound (TVUS) imaging or their combination did not improve the early detection rates of OVCA. Because, CA-125 is not specific for early OVCA and the traditional TVUS imaging cannot detect early OVCA due to its limited resolution [6]. Thus current detection limit of traditional TVUS imaging needs to be improved. MT-U/S can detect tumor associated changes expressed by the tumor epithelium or by the endothelium of tumor associated microvessels. The goal of this study is to develop and test the efficacy of IL-16-targeted MT-U/S imaging agent for the detection of early OVCA in association with its serum levels together with serum anti- NMP antibodies. This goal is being achieved by two specific aims. IL-16-targeted MT-U/S imaging agent was developed and tested in Aim-I while Aim-2 will test the efficacy of this newly developed IL-16-targeted imaging agent in a prospective study using laying hens, a preclinical model of spontaneous OVCA.

BODY: the research accomplishments associated with each task outlined in the approved Statement of Work.

The approach is to detect OVCA at early stage by contrast enhanced ultrasound imaging targeting IL-16 expressing ovarian tumor epithelium. The hypothesis of this project is that *OVCA at early stage can be detected using molecular (IL-16) - targeted ultrasound (MT-US) imaging in association with serum IL-16 levels and anti-NMP antibodies*. This hypothesis is being tested with the following tasks.

Task 1. Molecular targeted ultrasound (MT-U/S) imaging of hen ovarian tumor associated neo-angiogenesis (TAN) (months 1-11)

1a. Scanning of 150 hens to select hens with ovarian tumor associated abnormalities (month 1-3).

1. Hens were monitored for 1 month, egg laying rates were recorded and hens with normal or abnormal ovaries suspected for ovarian tumors were selected.
2. IL-16-targeted microbubble contrast agents were developed by conjugating anti-chicken IL-16 antibodies with microbubbles from Targeson, Inc., San Diego, CA.
3. Hens were scanned with pre-targeted and post-targeted gray scale and Doppler imaging using targeted contrast enhanced imaging agents.

4. Blood samples were collected, serum samples were separated and stored in -80°C until further use
5. Following scan, all hens were euthanized, ovarian tissues were collected and processed for paraffin, frozen and molecular biological studies including proteomics and gene expression analysis.

1b. Ovarian Histopathology and Immunohistochemistry:

1. OVCA diagnosis by routine histopathology: Paraffin blocks of a portion of the harvested ovaries were made and 5 µm thick representative sections of all blocks of each ovary were cut and stained with hematoxylin & eosin and examined under a light microscope for the presence or absence of tumor.
2. Paraffin and frozen sections from ovarian blocks were made and immunohistochemical detection of IL-16 expression by tumor epithelium (using similar anti-chicken IL-16 antibodies used for targeted imaging) and stromal cells as well as detection of neo-angiogenic microvessels (using anti-Smooth muscle actin antibodies) were performed.
3. Sections were analyzed by counting of immunopositive IL-16 expressing cells and SMA-expressing microvessels and the correlation between the immunohistochemical markers and histopathological disease status were examined.

1c. Biochemical and molecular biological studies, and processing of archived ultrasound imaging data.

1. Serum prevalence of anti-NMP antibodies and IL-16 levels were determined immunoassay (ELISA).
2. Expression of IL-16 mRNA (RT-PCR) and protein (1 and 2-dimensional Western blotting) by hen ovaries with or without tumors were examined.
3. Archived ultrasound images were examined off-line, ovarian tumor associated changes in gray scale intensity and Doppler indices were determined and IL-16 targeted imaging parameters associated with early stage OVCA were determined.
4. Correlation among IL-16 targeted ultrasound imaging parameters with serum prevalence of anti-NMP antibodies and IL-16 levels detective of early stage OVCA were determined. Diagnostic level of serum IL-16 and MT-U/S imaging parameters indicative of early stage ovarian cancer with reference to histopathological observation for use in Specific Aim 2 were determined.

Milestone for Task 1: *Association of serum anti-NMP antibodies with OVCA, serum levels of IL-16 and IL-16-targeted MT-U/S imaging indices detective of ovarian tumor at early stage was determined.*

Detailed Reports on the Accomplishments:

Specific Aim 1: Ovarian molecular (IL-16) targeted ultrasound (MT-US) imaging will differentiate hens with early stage OVCA from hens with normal ovaries.

Animals:

Three years old White Leghorn hens with low egg laying rates and normal egg laying rates were selected from a flock raised with identical care and management. Hens were scanned with traditional TVUS and Doppler ultrasound imaging followed by IL-16-targeted contrast enhanced ultrasound imaging. Blood samples were collected from the brachial vein before sonographic examination and serum samples were separated and stored at -80°C until further use. Ovarian tissues were collected following euthanasia immediately after MT-U/S imaging and processed for histopathology, immunohistochemistry, proteomics and gene expression analysis. Serum samples were used to determine the prevalence of anti-NMP antibodies as well as concentration of IL-16.

IL-16 targeted contrast enhanced imaging agents

IL-16-targeted imaging agents were prepared by conjugating anti-chicken IL-16 antibodies with Targetster® containing microbubbles (Targeson, Inc San Diego, CA). The agent remains acoustically active for 5-15 minutes. Agents are administered as an intravenous bolus injection. Microbubbles preparation, ligand conjugation, characterization of labeled microbubbles were similar to those reported earlier [7].

Contrast enhanced molecular (IL-16) targeted ultrasound imaging (MT-U/S):

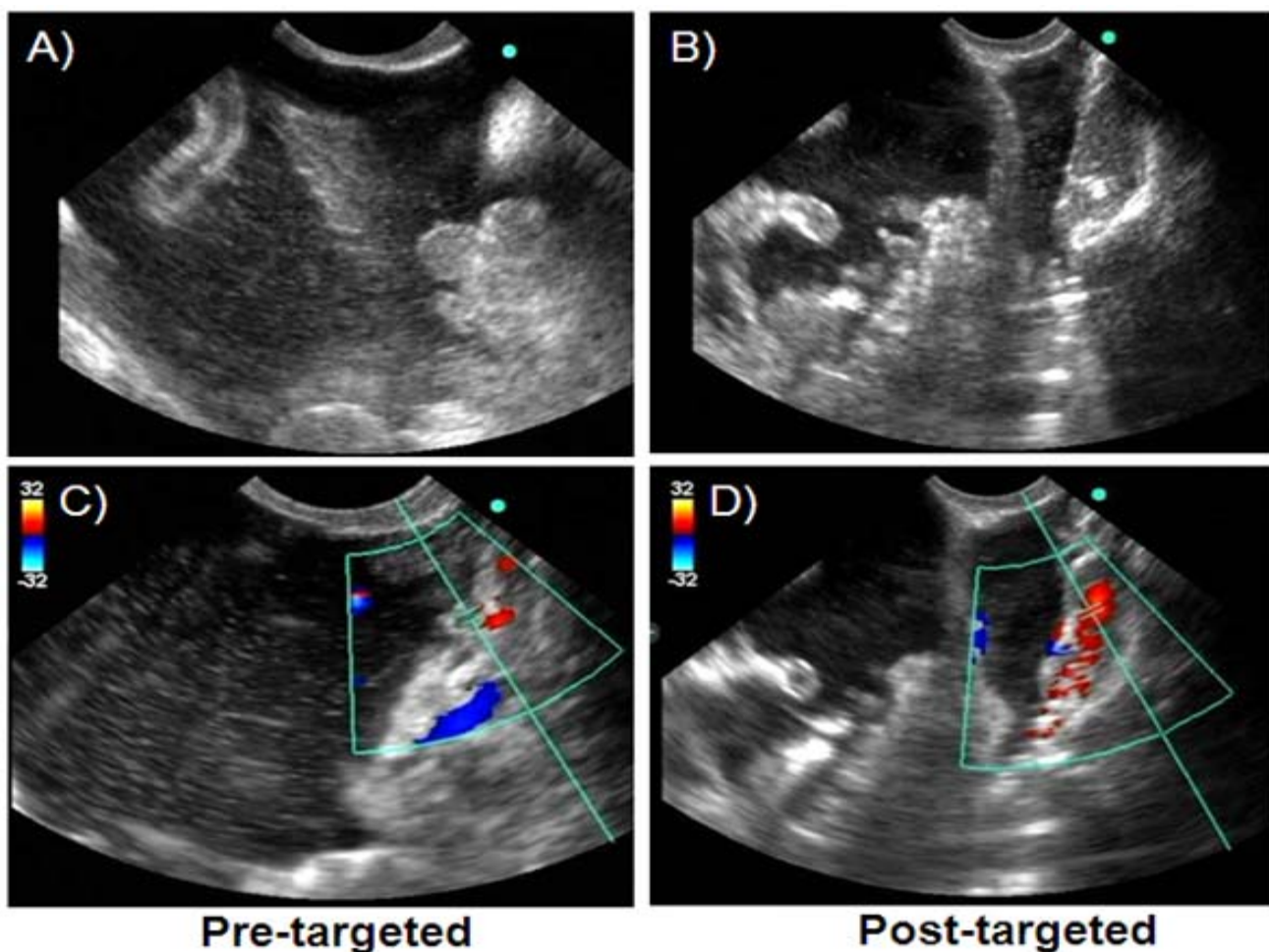
Pre-targeted imaging: Pre-targeted ultrasound scanning of hen ovaries was performed prior to the injection of IL-16 targeted imaging agents using similar procedure as reported earlier with an instrument attached to a 5- to 7.5-MHz endovaginal transducer (MicroMaxx; SonoSite, Inc, Bothell, WA)[8, 9]. Briefly, hens were immobilized and gently held by an assistant, the transducer probe was inserted transvaginally and 2-dimensional (2D) transvaginal gray scale and pulsed Doppler imaging were performed. Morphologic features were determined by gray scale imaging and vasculature features were determined by Doppler imaging. Doppler indices, resistive index (RI: [systolic velocity – diastolic velocity]/systolic velocity) and the pulsatility index (PI: [systolic velocity – diastolic velocity]/mean) calculated automatically by the software in-built in the machine. Minimum two separate Doppler images for each ovary were taken and the lower RI and PI values were used for analysis. All images were processed and digitally archived.

IL-16-targeted ultrasound imaging:

Following pre-targeted imaging, hens were injected with 10uL/Kg body weight of IL-16 targeted microbubbles at the brachial vein as reported earlier. Following the injection of ovarian IL-16-targeted microbubbles in a similar manner with identical mechanical settings as described above for pre-targeted imaging. The same pre-targeted and adjacent areas were imaged using similar procedure as reported earlier report [10]. Targeted microbubbles were accumulated at the target sites (tumor epithelium) after 5-7 min from the arrival, and unbound free microbubbles were washed out. All images were archived digitally in a still format as well as in real-time clips (10-15 minutes for each hen). Signals due to binding of microbubbles with their targets were visually evaluated online during the scanning period and off-line afterwards by reviewing the archived still images and video clips. The arrival-time of contrast agents (intervals from injection of the microbubbles containing contrast agents to its visual observation [in seconds]) in the ovaries with or without tumor was recorded in real time. After review of the complete clip, the region of interest (ROI) was selected. A ROI comprising the tumor was selected and the average image intensity (in pixel values) was determined using

computer assisted software (Microsuite™ version Five, Olympus America, Inc., Canter Valley, PA). Post-targeted imaging intensity was compared with the intensity of the pre-targeted imaging. The pixel intensities of ROI predictive of OVCA were determined. In addition, RI and PI values from post-targeted imaging were calculated.

Results: Pre- and post-targeted representative sonograms of an ovary predicted to have tumor together with its corresponding gross presentation are shown in **Figures 1 and 2**. Scanning of normal ovaries in healthy hens with low egg laying rates (data not shown) detected a couple of well developed preovulatory follicles together a few small growing follicles. Of all hens scanned, 23 were suspected to have solid mass. Compared with pre-targeted scanning, IL-16-targeted contrast enhanced imaging improved the visualization of ovarian tumor



masses in these 23 hens on gray scale. All of these hens were

Figure 1: Enhancement of ultrasound signal intensity by IL-16 targeted molecular ultrasound imaging of hen ovarian tumors. A) Pre-targeted gray scale sonogram depicting a suspected ovarian mass. B) Gray scale sonogram of the same ovary 7 min after the injection of IL-16 targeted imaging agents (microbubbles). Compared with pre-targeted sonogram, IL-16 targeted imaging agents increased ultrasound signal intensity remarkably in post-targeted imaging. The tumor showed septa of tissue mass during targeted imaging. C) Pre-targeted Doppler sonogram. D) Post-targeted Doppler sonogram. More microvessels were detected in post-targeted Doppler imaging than pre-targeted Doppler imaging.

categorized as "hens with suspected ovarian cancer". Compared with pre-targeted ROI (either from normal or ovaries with suspected ovarian tumors), the signal intensities were higher in post-targeted imaging. In normal healthy hens with low egg laying rates, the mean signal intensity of IL-16-targeted imaging was $2,668,772 \pm$

376,716 (mean \pm SD) pixels and it was significantly higher in hens in which tumor masses was seen to be limited to a part of the ovary (early stage) 6386746 ± 2036507 (mean \pm SD) pixels. The signal intensities increased further in hens predicted to have late stage OVCA (with solid masses in the organs of peritoneal cavity, in addition to the ovary and containing profuse ascites) $6,799,295 \pm 1,145,487$ (mean \pm SD) pixels. Compared with normal ovaries, more blood vessels were seen in hens suspected to have OVCA by pulsed color Doppler ultrasound imaging. The mean pre-targeted RI values for hens with normal, with small or large ovarian masses were 0.62 ± 0.03 , 0.49 ± 0.07 and 0.36 ± 0.03 , respectively. The RI values were found to be decreased in all hens to 0.55 ± 0.04 (normal), 0.45 ± 0.06 (early stage) and 0.23 ± 0.07 (late stage), respectively, in post-targeted color Doppler ultrasound imaging. Similar patterns were also observed for PI values.

Ovarian histopathology and immunohistochemical studies:

IL-16-targeted contrast enhanced ultrasonographic diagnosis of suspected OVCA were confirmed by gross examinations following euthanasia. Ovarian morphology including the ovarian follicles and their sizes, oviducts, presence of solid mass in the ovary, levels of tumor metastasis, OVCA stages and accompanying ascites, were recorded and tissues were processed as mentioned above. Tumor types were determined by routine hematoxylin & eosin staining (H&E) of paraffin sections (**Figure 2**).

Staging of ovarian tumors was performed as reported previously [11]. As observed during targeted imaging, late stage OVCA (n = 16 hens including 7 serous, 6 endometrioid, 3 mucinous) was associated with moderate to profuse ascites and metastasized to peritoneal and abdominal organs. Tumors in early stage OVCA (n = 7 including 4 serous, 2 endometrioid, 1 mucinous) were limited to the ovary with no or very little ascites.

Immunohistochemical detection of markers of OVCA or ovarian tumor associated neoangiogenesis: Paraffin sections of normal or tumor ovaries were immunostained for the detection of IL-16 expressing cells and SMA-expressing microvessels using specific antibodies and the frequencies of the immunopositive cells and vessels were counted and analyzed as reported previously [12, 13, 5]. Differences in the frequency of these markers between normal and hens with OVCA were considered significant when the $P < 0.05$.

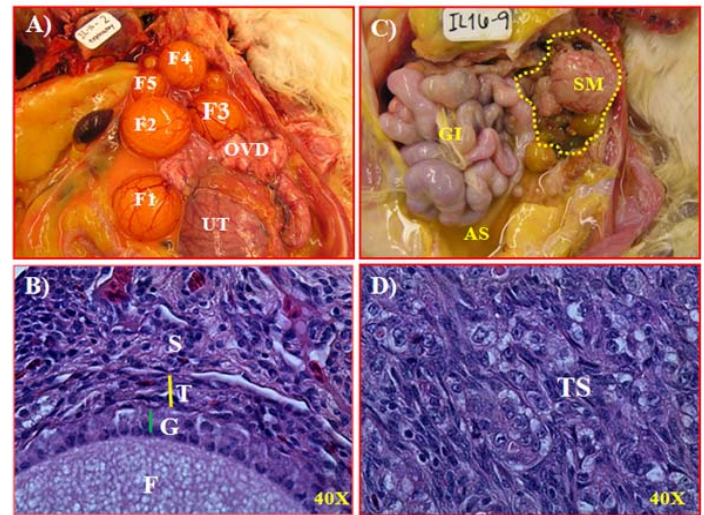


Figure 2: Gross and microscopic presentation of normal and tumor ovaries confirming the predictions of IL-16 targeted molecular ultrasound imaging in hens. A-B) A normal hen ovary, as predicted by targeted imaging, had a hierarchy (F1-F5) of developing large preovulatory follicles (A) while a cortical follicle is seen embedded in the stroma (B). C-D) Spontaneous ovarian tumor in a hen predicted to have solid mass (SM) in the ovary during IL-16 targeted ultrasound imaging. As predicted during imaging, the tumor was accompanied with profuse ascites (AS) and coiled gastrointestinal (GI) tract. Histological examination confirmed the tumor was a serous ovarian adenocarcinoma. F=follicles, G=granulosa layer, OVD=oviduct, S=stroma, T=theca layer, TS=tumor stroma.

Detection of IL-16 expressing cells: Very few IL-16 expressing cells were seen in the ovarian stroma and the follicular theca layer of normal healthy hens with low egg laying rates (**Figure 3A**). Compared with normal hens many IL-16 expressing cells were localized in hens with OVCA (**Figure 3B-C**). The frequency of stromal IL-16 expressing cells was significantly ($P<0.05$) higher in hens with early stage OVCA (mean \pm SD= 21.56 ± 6.65 in $20,000\mu\text{m}^2$ of tumor tissue) than in normal hens (9.68 ± 6.65 in $20,000\mu\text{m}^2$ of ovarian stromal tissue), and increased further in hens with late stage of OVCA (28.64 ± 5.25 in $20,000\mu\text{m}^2$ of tumor tissue) (**Figure 4, top panel**).

Changes in serum IL-16 levels in association with OVCA development:

Concentrations of IL-16 in serum of normal and OVCA hens were determined by immunoassay using anti-Chicken IL-16 Vetset™ ELISA Kit (Kingfisher Biotech, St. Paul, MN). 96-well ELISA plates pre-coated with anti-Chicken IL-16 antibodies were used and chicken IL-16 protein was used as standard according to the manufacturer's instructions as reported earlier [5]. All standards and serum samples were run in duplicate. Optical density (OD) value for each well was read at 450nm in a plate reader (Thermomax;

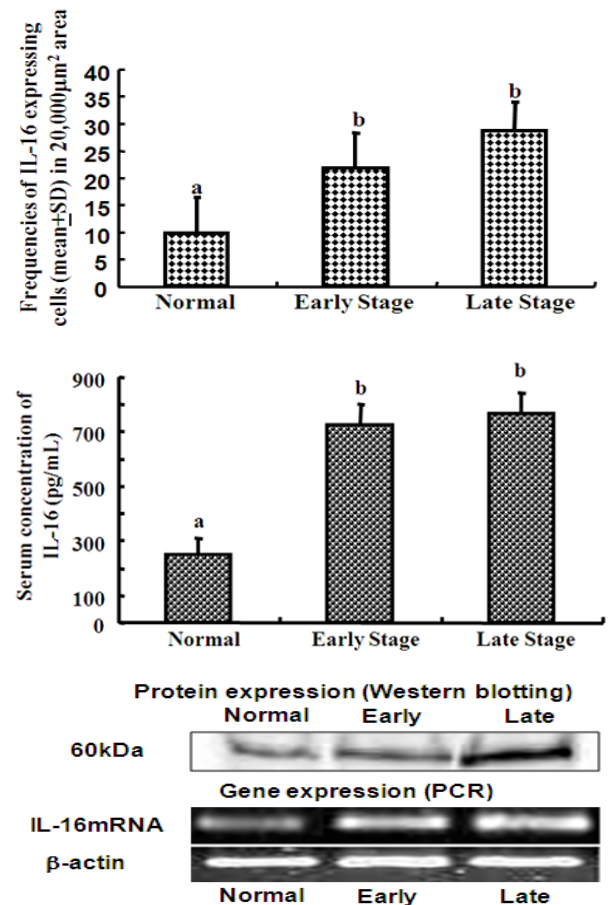


Figure 4. Changes in the frequency of IL-16 expressing cells and its serum level in association with ovarian cancer (OVCA) development and progression. **Top panel:** Frequency of IL-16 expressing cells increased significantly with tumor initiation and progression to late stages. **Middle panel:** Concentration of serum IL-16 increased significantly in association with OVCA development and progression. **Bottom panel:** Molecular studies showed changes in tissue expression and serum levels of IL-16 during OVCA development was associated with increase in IL-16 gene and protein expression. Bars with different letters are significantly ($P<0.01$) different.

Figure 3. Immunohistochemical detection of changes in IL-16 expression in association with ovarian cancer (OVCA) development and progression in hens. A) Section of a normal hen ovary showing few IL-16 expressing cells in the ovarian stroma (S) and the follicular (F) theca (T). B and C) Sections of tumor ovaries at early (B) and late stage of OVCA (C). Compared with normal ovary, many IL-16 expressing cells are seen in OVCA hens. TG=tumor gland, TS=tumor stroma, Arrows indicate the examples of IL-16 expressing cells.

Sunnyvale, CA). A standard curve was made by plotting the OD values of the standards against their concentrations. Serum IL-16 concentrations were determined with reference to the standard curve as per manufacturer's instruction using a software program (Softmax Pro, version 1.2.0, software; Molecular Devices, Sunnyvale, CA).

Compared with normal hens (mean \pm SD = 248.95 ± 62.73 pg/mL), the mean concentration of serum IL-16 was significantly higher ($P < 0.0001$) in hens with early (721.31 ± 77.72 pg/mL) stage OVCA. The concentration of IL-16

increased further in hens with late stage OVCA (764.20 ± 77.67 pg/mL) (**Figure 4, middle panel**). The differences in serum IL-16 levels were not significant between the early and late stages OVCA as well as among the different histological subtypes of OVCA.

IL-16 gene expression during OVCA development: Changes in IL-16 mRNA expression was examined in normal ovaries or ovaries with tumors.

IL-16 mRNA expression by normal ovaries or ovaries with tumor in hens: IL-16 mRNA expression was assessed by semi-quantitative RT-PCR as reported previously [14, 5]. Chicken-specific IL-16 primers were designed (Oligopredictor Designer software, Invitrogen, Carlsbad, CA) using IL-16 sequence from the NCBI (GeneBank: NM_204352.3). The forward primer was 5-TCTCTGCTTTCCCTGAA-GA and the reverse primer was 5-GTCCATTGGGAAACACCT-TG located between exons 4 and 6. β -actin was used as the endogenous control with a forward primer of TGCGTGACATCAAGGAGAAG and a reverse primer of ATGCCAGGGTACATTGTGGT. The expected base pair size for the IL-16 amplicon was 199 bp and for β -actin was 300 bp. PCR amplicons were visualized in a 3% agarose gel (Pierce/Thermo Fisher, Rockford, IL USA) in TAE buffer and stained with ethidium bromide. The image was captured using a ChemiDoc XRS system (Bio-Rad, Hercules, CA).

Ovarian homogenates from normal hens showed an weak expression for IL-16 mRNA. In contrast, OVCA hens showed a strong amplification signal for IL-16 mRNA (**Figure 4, bottom panel**) and no significant difference was observed in IL-16 mRNA expression among different histological subtypes of OVCA as well as early and late stage OVCA. Thus, enhanced IL-16 mRNA expression in OVCA hens supports the increased expression serum levels of IL-16 observed in hens with OVCA. **Overall**, the results of the immunohistochemical studies suggest that the ovarian malignant epithelium expressed IL-16 and IL-16 targeted microbubbles bound with these IL-16 expressing malignant epithelial cells which might be a reason of increased signal intensity observed during the targeted imaging.

Detection of ovarian tumor associated neoangiogenic microvessels:

Ovarian tumor associated neoangiogenic (TAN) microvessels were detected by anti-SMA antibodies. As reported earlier, these SMA-expressing microvessels were immature and appeared to be leaky with a discontinuous smooth muscle layer surrounding them. SMA-expressing microvessels were localized in the ovarian stroma and follicular theca of normal follicles as well as in tumor stroma in hens with OVCA.

Expression of SMA by TAN vessels: SMA-expressing microvessels were localized at the spaces between tumor glands (tumor vicinity, **Figure 5**). The frequencies of SMA-expressing microvessels were significantly ($P < 0.001$) higher in hens with early stage OVCA (mean \pm SD= 15.60 ± 3.62 in $20,000\mu\text{m}^2$ of tumor tissue) than in normal hens (2.9 ± 1.31 in $20,000\mu\text{m}^2$ of ovarian stromal tissue) and increased further in hens with late stage of OVCA (23.65 ± 2.45 in $20,000\mu\text{m}^2$ of tumor tissue) ($P < 0.05$) (**Figure 6**). Significant differences in the frequencies of SMA-expressing microvessels were not observed among different histological sub-types of

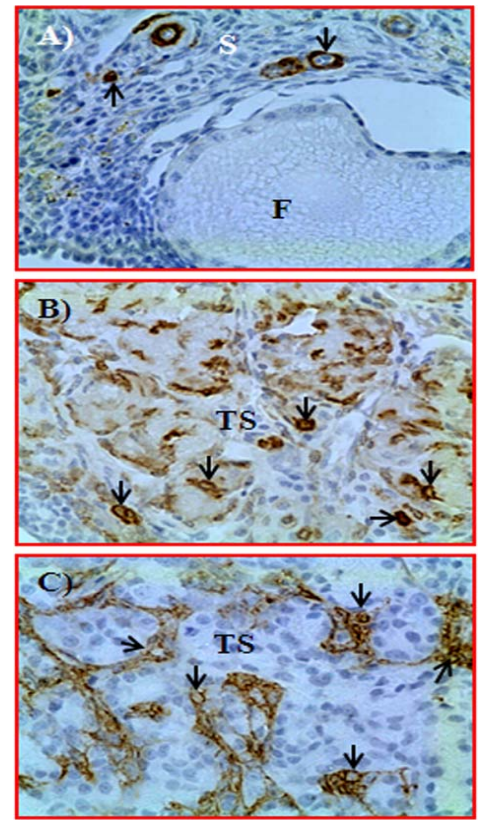


Figure 5. Immunohistochemical detection of changes in smooth muscle actin (SMA, a marker of angiogenic microvessels) expression in association with OVCA development and progression in hens. A) Section of a normal hen ovary showing few SMA-expressing microvessels in the stroma (S) and the follicular (F) theca. B and C) Sections of tumor ovaries at early (B) and late stages of OVCA (C). Compared with normal ovary, many SMA-expressing microvessels are seen in OVCA hens. TS=tumor stroma, Arrows indicate examples of SMA-expressing vessels. F= follicle. 40X.

ovarian tumors in hens. These results supports the notion that tumor associated neo-angiogenesis is an early event in OVCA development and progression. RI and PI values of early stage OVCA hens with highest SMA-expressing microvessels was considered as the diagnostic value of ovarian TAN at early stage.

Biochemical and molecular biological Studies:
Serologic and Proteomic detection of malignant nuclear transformation: Malignant nuclear transformation accompanies with the shedding of nuclear matrix proteins in circulation which resulted in the production of anti-NMP antibodies. Serum prevalence of anti-NMP antibodies were determined by immunoassay and confirmed by 2-dimenssional Western blotting (2D-WB). We are the first to show the prevalence of serum anti-NMP antibodies in OVCA hens [3] reported earlier.
Detection of anti-NMP antibodies in serum of OVCA hens:
 Normal or tumor ovarian NMPs from healthy hens with low egg

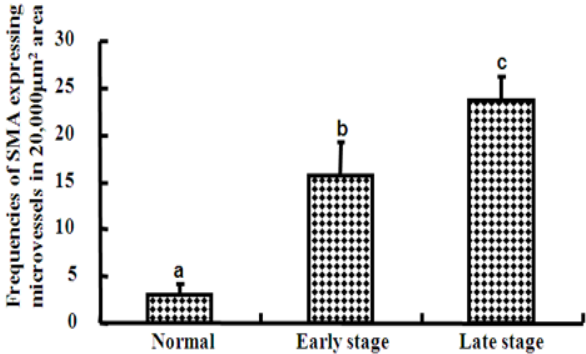


Figure 6. Changes in the frequency of SMA-expressing microvessels in association with OVCA development and progression. The frequency of SMA-expressing microvessels increased significantly with tumor development and increased further as the OVCA progressed to late stages. Bars with different letters are significantly ($P<0.01$) different.

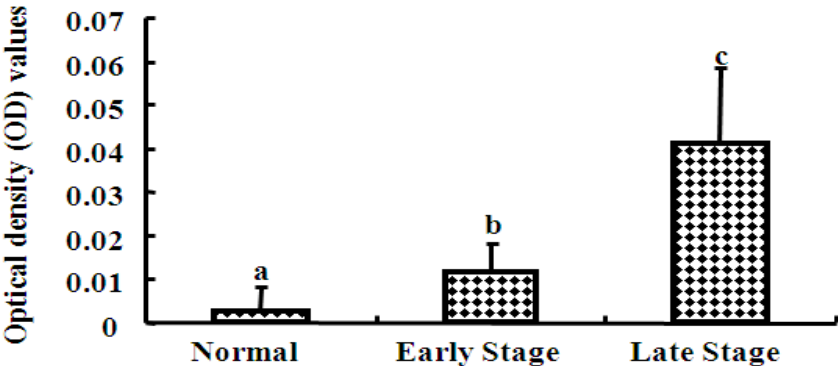
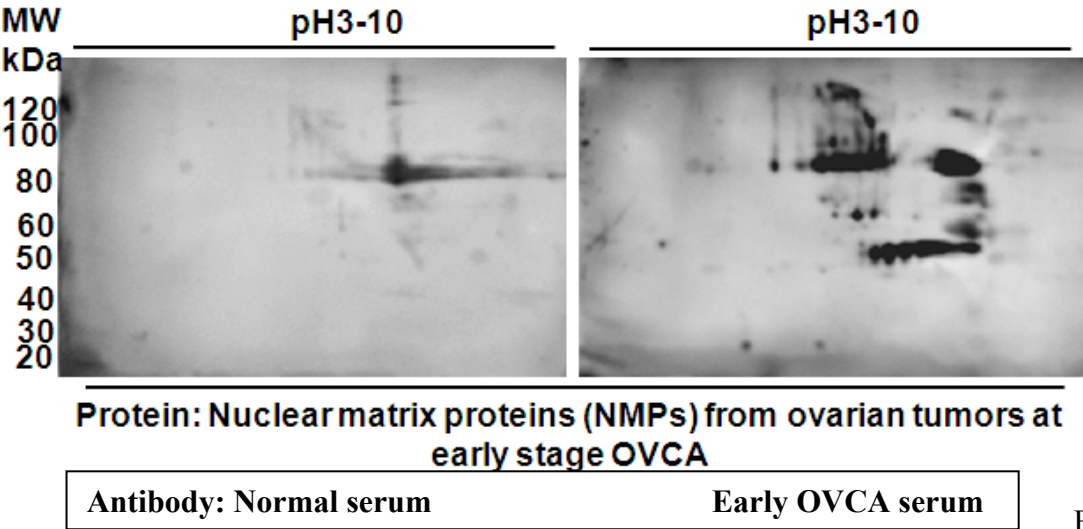


Figure 7. Top panel: Proteomic detection of immunoreactive nuclear matrix protein antigens associated with OVCA development using serum of OVCA hens. NMPs from hens with early OVCA were electrophoresed and blotted in nitrocellulose membrane and immunoreactions against serum anti-NMP antibodies were detected by 2-Dimensional Western blotting (2D-WB). Compared with normal serum (left panel), many immunoreactive NMPs were detected by OVCA serum (right panel). **Bottom panel:** Changes in serum reactivity (measured as optical density, OD) against ovarian NMPs in association with prevalence of anti-NMP antibodies. Compared with normal, OD values were significantly high ($P<0.001$) in hens with early and late stages of OVCA. Bars with different letters are significantly different.

laying rates or OVCA hens were extracted as reported previously [3,4]. ELISA (96-well) plates were coated with extracted NMPs and the immunoreactivity of normal or tumor serum samples against or tumor NMPs were determined as reported earlier [15]. Each serum sample was assayed in duplicate and the plates were read at 405nm in an ELISA plate reader (Softmax Pro, version 1.2.0, software; Molecular Devices, Sunnyvale, CA). Serum samples from young healthy normally laying hens were used as negative control for the prevalence of anti-NMP antibodies. Representative serum samples with or without anti-NMP antibodies were examined by 2D-Western blot and confirmed the results of immunoassays. Strong immunoreactivity of tumor serum against tumor NMP proteins showed multiple

immunoreactive spots of different sizes and pH while a few spots with weak immunoreactivity were seen against normal serum (**Figure 7, top panel**). Hens with early stage OVCA had significantly high OD values than normal hens (**Figure 7, bottom panel**). Positive immunoreactivity for the prevalence of anti-NMP antibodies in serum was considered when optical density (OD) values were higher than the control mean + 2SD (cut-off value or 5 fold higher than the OD values of normal hens). Thus these results confirmed previous observation that anti-NMP antibodies are associated with malignant ovarian transformation and may be used as a surrogate marker of OVCA.

Diagnostic indices for early detection of OVCA: Overall, IL-16 targeted imaging intensity diagnostic of early stage OVCA mentioned below is established with reference to histopathology and ovarian expression of IL-16 and SMA.

- a. *IL-16-targeted contrast enhanced imaging signal intensities:* diagnostic of early stage OVCA: 45,000pixels or higher
- b. *Pulsed Doppler indices associated with early stage OVCA:* from contrast enhanced ultrasound molecular imaging: RI = 0.45 or lower
- c. *Serum IL-16 level Diagnostic of early stage OVCA:* approximately 500 pg/mL (based on the lowest values of hens with early OVCA as well as 2 fold higher than the IL-16 levels of normal hens) or higher based on corresponding histopathological diagnosis of ovarian tumors.

KEY RESEARCH ACCOMPLISHMENTS:

- This is the first study showing targeting of tumor epithelium directly by in vivo ultrasound imaging.
- This is also a first study showing a link between longstanding unresolved inflammation and ovarian malignancy mediated by IL-16 (a pro-inflammatory cytokine).
- Enhancement of signal intensity and ability of early stage OVCA detection of traditional TVUS scanning by IL-16- targeted molecular imaging agents.
- Ultrasound prediction of tumor related over-expression of ovarian IL-16 confirmed by immunohistochemical, proteomic and gene expression analysis.
- This study also showed the potential of serum IL-16 levels as a novel marker of early stage OVCA, may be used together with serum anti-NMP antibodies (a marker of malignant nuclear transformation).
- This study revealed IL-16 as a novel therapeutic target for preventing OVCA.

REPORTABLE OUTCOMES:

Presentation: Abstract published and presented: (*copy attached*)

1. Yellapa A, Bitterman P, Abramowicz JS, Bahr JM, Basu S, Sharma S and Barua A. IL-16 in association with VEGFR-2 detect early tumor associated changes in the ovary. [abstract]. In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6-10; Washington, DC. Philadelphia (PA): AACR; Cancer Res 2013; 73(8 Suppl): Abstract nr 4641. doi:10.1158/1538-7445.AM2013-4641

Manuscript: One manuscript is under preparation.

CONCLUSION:

Accomplishment of Aim 1 suggest that IL-16-targeted ultrasound molecular imaging probes bound with ovarian malignant epithelium and enhanced the imaging signal intensities in laying hen model of spontaneous ovarian cancer (OVCA). This study also confirms previous findings that anti-NMP antibodies are prevalent in association with ovarian tumor development. Increase in serum levels of IL-16 was associated with the increased OVCA related imaging indices in hens. IL-16-targeted ultrasound imaging indices and IL-16 serum levels detective of OVCA determined in Aim 1 will be tested in Aim 2 for their efficacy in detecting OVCA at early stage.

REFERENCES

- [1] Siegel, R., D. Naishadham and A. Jemal (2012). "Cancer statistics, 2012." CA Cancer J Clin **62**(1): 10-29.
- [2] Ries, L. A. (1993). "Ovarian cancer. Survival and treatment differences by age." Cancer **71**(2 Suppl): 524-9.
- [3] Barua, A., Q. T, B. P, B. JM, B. S, E. SL and A. JS (2012). "Molecular targeted imaging of vascular endothelial growth factor receptor (VEGFR)-2 and anti-NMP autoantibodies detect ovarian tumor at early stage " In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2012;72(8 Suppl):Abstract nr 2455. doi:1538-7445.AM2012-2455
- [4] Yu, E., H. Lee, W. Oh, B. Yu, H. Moon and I. Lee (1999). "Morphological and biochemical analysis of anti-nuclear matrix protein antibodies in human sera." J Korean Med Sci **14**(1): 27-33.
- [5] Yellapa, A., J. M. Bahr, P. Bitterman, J. S. Abramowicz, S. L. Edassery, K. Penumatsa, S. Basu, J. Rotmensch and A. Barua (2012). "Association of interleukin 16 with the development of ovarian tumor and tumor-associated neoangiogenesis in laying hen model of spontaneous ovarian cancer." Int J Gynecol Cancer **22**(2): 199-207.
- [6] Moore, R. G. and R. C. Bast, Jr. (2007). "How do you distinguish a malignant pelvic mass from a benign pelvic mass? Imaging, biomarkers, or none of the above." J Clin Oncol **25**(27): 4159-61.
- [7] Anderson, C. R., J. J. Rychak, M. Backer, J. Backer, K. Ley and A. L. Klibanov (2010). "scVEGF microbubble ultrasound contrast agents: a novel probe for ultrasound molecular imaging of tumor angiogenesis." Invest Radiol **45**(10): 579-85.
- [8] Barua, A., J. S. Abramowicz, J. M. Bahr, P. Bitterman, A. Dirks, K. A. Holub, E. Sheiner, M. J. Bradaric, S. L. Edassery and J. L. Luborsky (2007). "Detection of ovarian tumors in chicken by sonography: a step toward early diagnosis in humans?" J Ultrasound Med **26**(7): 909-19.
- [9] Barua, A., P. Bitterman, J. M. Bahr, M. J. Bradaric, D. B. Hales, J. L. Luborsky and J. S. Abramowicz (2010). "Detection of tumor-associated neoangiogenesis by Doppler ultrasonography during early-stage ovarian cancer in laying hens: a preclinical model of human spontaneous ovarian cancer." J Ultrasound Med **29**(2): 173-82.
- [10] Anderson, C. R., X. Hu, H. Zhang, J. Tlaxca, A. E. Decleves, R. Houghtaling, K. Sharma, M. Lawrence, K. W. Ferrara and J. J. Rychak "Ultrasound molecular imaging of tumor angiogenesis with an integrin targeted microbubble contrast agent." Invest Radiol **46**(4): 215-24.

- [11] Barua, A., P. Bitterman, J. S. Abramowicz, A. L. Dirks, J. M. Bahr, D. B. Hales, M. J. Bradaric, S. L. Edassery, J. Rotmensch and J. L. Luborsky (2009). "Histopathology of ovarian tumors in laying hens: a preclinical model of human ovarian cancer." Int J Gynecol Cancer **19**(4): 531-9.
- [12] Bradaric, M. J., A. Barua, K. Penumatsa, Y. Yi, S. L. Edassery, S. Sharma, J. S. Abramowicz, J. M. Bahr and J. L. Luborsky (2011). "Sphingosine-1 phosphate receptor (S1p1), a critical receptor controlling human lymphocyte trafficking, is expressed in hen and human ovaries and ovarian tumors." J Ovarian Res **4**(1): 4.
- [13] Barua, A. and Y. Yoshimura (1999). "Effects of aging and sex steroids on the localization of T cell subsets in the ovary of chicken, *Gallus domesticus*." Gen Comp Endocrinol **114**(1): 28-35.
- [14] Hong, Y. H., H. S. Lillehoj, S. H. Lee, R. A. Dalloul and E. P. Lillehoj (2006). "Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections." Vet Immunol Immunopathol **114**(3-4): 209-23.
- [15] Barua, A., S. L. Edassery, P. Bitterman, J. S. Abramowicz, A. L. Dirks, J. M. Bahr, D. B. Hales, M. J. Bradaric and J. L. Luborsky (2009). "Prevalence of antitumor antibodies in laying hen model of human ovarian cancer." Int J Gynecol Cancer **19**(4): 500-7.

Cancer Research: April 15, 2013; Volume 73, Issue 8, Supplement 1
doi: 10.1158/1538-7445.AM2013-4641
Proceedings: AACR 104th Annual Meeting 2013; Apr 6-10, 2013; Washington, DC
© 2013 American Association for Cancer Research

Poster Presentations - Biomarkers of Drug Response

Abstract 4641: IL-16 in association with VEGFR-2 detect early tumor associated changes in the ovary.

Aparna Yellapa¹, Pincas Bitterman¹, Jacques S. Abramowicz¹, Janice M. Bahr², Sanjib Basu¹, Sameer Sharma¹, and Animesh Barua¹

¹Rush Univ. Medical Ctr., Chicago, IL;

²University of Illinois at Urbana-Champaign, IL.

Background: Ovarian cancer (OVCA) is a lethal malignancy of women with high rate of death among gynecological cancers. Due to the lack of an effective early detection test, OVCA in most cases are detected at late stage. Interleukin (IL)-16 is a cytokine involved in multiple immunopathobiological processes including inflammatory responses and production of proangiogenic cytokines. IL-16 is expressed by malignant epithelium and secreted in serum. Ovarian malignant transformation is followed by neovascularization and vascular endothelial growth factor receptor-2 (VEGFR-2) is a marker of tumor associated neovascularization (TAN). Thus, IL-16 and VEGFR-2 represent markers of OVCA at early stage may constitute an early detection test for OVCA.

Objectives: The goal of this study was to determine whether IL-16 expression is associated with ovarian malignant transformation and to examine association between serum IL-16 levels and VEGFR-2 expression by TAN vessels during ovarian tumor development.

Materials and methods: Normal (n =15) White Leghorn laying hens (3-4 years old) or hens with ovarian tumors (n=32) were selected by contrast enhanced transvaginal ultrasound scanning. Following serum collection, hens were euthanized, tissues were processed for routine histology, immunohistochemistry (IHC), protein and gene expression studies. Ovarian tumors were confirmed by gross morphology and microscopy. Expression of IL-16 by ovarian malignant epithelium and VEGFR-2 by ovarian TAN vessels were determined by IHC, immunoblotting and quantitative PCR. Serum IL-16 levels were determined by immunoassay. Correlation between serum IL-16 levels and frequencies of VEGFR-2 expressing microvessels were examined.

Results: The intensity of IL-16 expression by normal ovarian epithelium was very weak (n=15). In contrast, the staining intensity of IL-16 by malignant epithelium increased significantly ($p < 0.001$) in hens with early stage OVCA (n=15) and late stage OVCA (n=17). Compared with normal hens, serum levels of IL-16 were significantly higher in hens with early (n=15) and late stages (n=17) of OVCA ($P < 0.0001$). The expression of IL-16 protein and mRNA were stronger in malignant ovaries than normal ovaries. The population of VEGFR-2 expressing microvessels increased significantly ($p < 0.001$) in hens with early stage OVCA than normal hens ($p < 0.001$) and increased further in late stage OVCA. The increase in serum IL-16 was positively correlated with the population of VEGFR-2 expressing TAN vessels.

Conclusion: IL-16 expression by ovarian malignant epithelium and its serum levels are associated with ovarian malignant transformation and correlated with VEGFR-2 expression. Thus IL-16 may be a useful serum marker and in combination with VEGFR-2 may detect OVCA at early stage. These findings will facilitate clinical studies to establish a non-invasive early OVCA detection test. Support: DOD Award # W81XWH-12-1-0460.

Citation Format: Aparna Yellapa, Pincas Bitterman, Jacques S. Abramowicz, Janice M. Bahr, Sanjib Basu, Sameer Sharma, Animesh Barua. IL-16 in association with VEGFR-2 detect early tumor associated changes in the ovary. [abstract]. In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6-10; Washington, DC. Philadelphia (PA): AACR; Cancer Res 2013;73(8 Suppl):Abstract nr 4641. doi:10.1158/1538-7445.AM2013-4641